Evaluation of Solid-Phase Microextraction for the Isotopic Analysis of Volatile Compounds Produced during Fermentation by Lactic Acid Bacteria

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The use of solid-phase microextraction (SPME) coupled with isotope ratio mass spectrometry (IRMS) for the analysis of flavor compounds produced by lactic acid bacteria has been evaluated using both liquid and headspace sampling modes. Initially, it was necessary to optimize the conditions for the SPME extraction of flavors—diacetyl and acetoin—in standard aqueous solutions. The effects of salt, headspace versus liquid sampling, and coating phase were tested. Second, the suitability of the coupling of SPME and gas chromatography−combustion interface−IRMS (GC-C-IRMS) for the determination of $\delta^{13}C$ values was assessed. It is shown that neither the analyte concentration nor the period of fiber exposure has an effect on the $\delta^{13}C$ values. Finally, having verified that there are no matrix effects from the fermentation medium, it is reported for the first time that flavor compounds can be extracted directly from culture supernatant by SPME and their $\delta^{13}C$ values can be obtained by GC-C-IRMS.

Keywords: *SPME; GC-C-IRMS; lactic acid bacteria; flavor compounds*

INTRODUCTION

Lactic acid bacteria (LAB) are widely used in industrial food fermentation processes. This microbial metabolism is exploited in fermented meats (sausages, hams), in fermented vegetables (sauerkraut, cucumber), and especially in fermented dairy products (yogurts, cheeses, and butter). The end products of lactic fermentation confer the necessary protection against spoilage (acidification, bacteriocins), contribute to the desired flavors, and add to the texture (Law, 1981). Some LABs can cometabolize citric acid and glucose. This cometabolism leads to the production of flavor compounds that are especially important in fermented milk products, notably, diacetyl ("buttery" flavor) and acetoin (flavorless).

The ability of different strains to produce these compounds is variable, and satisfactory levels are often achieved only by the use of mixed fermentations. To follow this production necessitates the extraction and measurement of these aromatic compounds. Because of their polarity and volatility, their recovery by classical techniques is unsatisfactory, requiring a relatively large volume of solvent and leading to losses of products. To study the production of diacetyl and acetoin during the fermentation of glucose and citric acid by *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis*, a rapid and reliable method of recovering these flavor compounds from the fermentation broth is required. Formation of the flavor compounds is followed by gas chromatography coupled via a combustion interface with an isotope ratio mass spectrometer (GC-C-IRMS) (Brand, 1996), which measures very small differences in the ^{13}C content of the volatile flavor compounds. Using this technique, the origin of these compounds can be quantitatively traced to either glucose or citric acid.

A stringent requirement of GC-C-IRMS is that the sample preparation does not lead to an isotopic fractionation or to an isotopic fractionation for which a correction cannot be applied. While the GC-C-IRMS analysis of acetoin is possible following its extraction with diethyl ether, this approach is tedious and prone to isotopic fractionation. Furthermore, the GC injection mode may also be a limitation to the technique. In splitless injection mode, due to its volatility, the more important flavor compound, diacetyl, is not sufficiently separated from the solvent tail to measure its isotopic content, and only the acetoin may be analyzed. In the split injection mode, the division applied by the split valve tends to lead to irreproducible isotopic fractionation for both compounds. Consequently, with organic extraction of diacetyl and acetoin, GC-C-IRMS analysis is difficult.

The recently developed method of solid-phase microextraction (SPME) integrates sampling, extraction, concentration, and sample introduction into a chromatography system in a single step (Arthur and Pawliszyn, 1990; Arthur et al., 1992b). In SPME, a fine fused silica capillary is coated with a stationary phase, the adsorbant properties of which can be varied depending of the stationary phase used. The fiber is placed into the matrix (liquid, solid, or gaseous) containing the analytes, from which they are directly adsorbed. The immediate advantages of the method (for reviews see Zhang et al., 1994; Eisert and Pawliszyn, 1997) are that no pre-extraction of the analytes is required, the sample being taken directly from the fermentation medium, and that a degree of selectivity can be obtained by the choice of the most appropriate fiber coating.

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Two independent studies have recently shown that SPME-GC analysis of volatile metabolites produced by LAB is possible (Vergnais et al., 1998) and that the coupling of SPME to GC-C-IRMS is feasible (Dias and Freeman, 1997). Moreover, the latter reference indicates that, at least from model solutions, the fractionation in $\delta^{13}C$ observed is small and consistent. In the present paper, it is shown, for the first time, that SPME-GC-C-IRMS can effectively be applied to the analysis of volatile metabolites in a fermentation medium. In the first instance, the extraction of the flavor compounds—diacetyl and acetoin—from aqueous culture supernatant and their analysis in GC-C-IRMS is reported. The development of this methodology indicates that, despite the potential problems due to the phase transition (Dias and Freeman, 1997), SPME-GC-C-IRMS can also be used to measure the ^{13}C content of the volatile flavor compounds by headspace sampling.

MATERIALS AND METHODS

Optimization of SPME. *Preparation of Test Solutions*. To evaluate SPME extraction of flavor compounds, an aqueous solution of diacetyl (1.16 mM) and acetoin (1.70 mM) was prepared in distilled water, aliquoted, and stored frozen. Standard compounds were purchased from Fluka (L'Isle d'Abeau Chesnes, France).

General SPME Procedure. The SPME device was purchased from Supelco (L'Isle d'Abeau Chesnes, France). Five different fiber coating phases were tested: poly(dimethylsiloxane) (PDMS, 100 μm); poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB, 65 μm); carbowax/divinylbenzene (CW/DVB, 65 μm); carboxene/poly(dimethylsiloxane) (CAR/PDMS, 75 μm); and polyacrylate (PA, 85 μm). Two sampling methods were investigated. For liquid sampling (LS), the fiber was inserted into a 40-mL glass vial containing 30 mL of sample saturated with salt (NaCl), thermostated at 30 °C, and left in the liquid during a defined exposure time under vigorous magnetic stirring. Then, the fiber was retracted and directly transferred to the injection port of a GC. For headspace sampling (HS), 25 mL of sample saturated with salt (NaCl) was placed in a 40-mL glass vial thermostated at 30 °C. Prior to the analysis, the headspace above the sample was left to equilibrate for 1 h under vigorous magnetic stirring. Then, the fiber was inserted into the vial and retained above the stirred liquid during a defined exposure time. After the defined exposure time, the fiber was retracted and directly transferred to the injection port of a GC.

Instrumental Analysis. *GC Analysis.* The analytes adsorbed on the fiber coating phase were thermally desorbed in the inlet of a HP 6890 gas chromatograph (Hewlett-Packard, Les Ulis, France) equipped with an EPC split-splitless inlet, fitted with a pre-pierced septum for SPME (Thermogreen LB-2, Supelco) and with a special injection sleeve (0.75-mm i.d., Supelco). Detection was made by FID, and the data were acquired using a HP ChemStation system. The inlet temperature was held constant during the analysis (240 °C). For separation, a 30-m, 0.25-mm i.d., 0.25-μm film thickness HP—Innowax column (Hewlett-Packard) was used. The GC oven was programmed as follows: 60 °C held for 3.5 min, increased to 100 °C at 7 °C/min, and then increased to 220 °C at 15 °C/min and held at 220 °C for 2 min. Helium was used as carrier gas (flow rate 1.5 mL/min, constant flow). For thermal desorption, the SPME fiber remained in the injector for 3 min. The injector was operated in the splitless mode, the purge valve being opened after 5 min.

GC-C-IRMS Analysis. An identical GC was coupled with a combustion interface and a Finnigan Mat-Delta Plus isotope ratio mass spectrometer. The GC was fitted with a 60-m, 0.32-mm i.d., 0.5-μm film thickness Stabilwax column (Restek, France). The GC oven was programmed as follows: 50 °C initial oven temperature with a 3.5-min hold, increased to 220 °C at 10 °C/min, and 220 °C held for 2 min. The helium flow was held constant at 2.2 mL/min. Splitless injection mode was used, the purge valve being opened after 5 min.

EA-IRMS (by Encapsulation). Reference $\delta^{13}C$ values for diacetyl and acetoin were determined by encapsulating 5 mg of compound in a silver capsule that was burned in the combustion interface at 960 °C in an oxygen-rich atmosphere (Brand, 1996). Then, the resulting CO_2 was carried by the helium flow into a Finnigan Mat-Delta E isotope ratio mass spectrometer.

Production of Bacterial Metabolites. *L. lactis* ssp. *lactis* biovar. *diacetylactis* strain B7/2147 was supplied by the National Collection of Lactic Acid Bacteria, Institute of Food Research, Norwich, UK. It was stored at −80 °C in M17 medium (Terzaghi and Sandine, 1975) with 15% glycerol and routinely cultured in sterile (30 min, 121 °C, 1 bar) M17 broth that contained per liter: tryptone 2.5 g, casein peptone 2.5 g, papain peptone 5 g, yeast extract 2.5 g, sodium glycerophosphate 19 g, magnesium sulfate 0.25 g, ascorbic acid 0.5 g. The bacteria were pre-cultured for 6 h in M17 with glucose (5 g/L) and citric acid (2.5 g/L) added by sterile filtration (0.2 μm, Millipore, Saint-Quentin, France). The fermentation was initiated by inoculating 200 mL of M17 in a 200-mL Duran bottle with 0.2 mL of pre-culture. The fermentation conditions were anaerobic (static), 30 °C, initial pH between 6.2 and 6.4 (HCl), and left to evolve freely during 16 h. Cultures were harvested after 16 h, and the supernatant was recovered by centrifugation (4500*g*, 10 min, 4 °C) and kept at −20 °C.

RESULTS AND DISCUSSION

Optimization of the SPME Procedure. *Effect of the Coating Phase.* Five types of fiber coating were evaluated for their extraction efficiency toward both diacetyl and acetoin by liquid sampling (Figure 1A). Only CAR/PDMS and PDMS/DVB showed a good adsorptive capacity toward both flavors. These two fibers were tested in headspace mode and showed a similar adsorption profile for acetoin and diacetyl from the gaseous phase (Figure 1B). PDMS/DVB showed approximately the same adsorptive capacity for the two flavors. In contrast, CAR/PDMS displayed a much higher overall adsorption capacity and adsorbed 4−5 times more diacetyl (the more volatile) than acetoin. This could prove advantageous when the aroma compounds are present at low concentrations, for example, at the beginning of the fermentation period. In addition, the better adsorptive capacity for diacetyl enhances the sensitivity toward this minor product. With concentrated samples, however, CAR/PDMS adsorption is too high, leading to saturation of the IRMS signal for diacetyl, even using a short exposure period. Under these conditions, the relative affinities for acetoin and diacetyl are such that, at the detection limit for acetoin, the diacetyl peak is already near saturation. Thus, both fibers show properties that are advantageous for metabolic studies.

Period of Adsorption. The equilibration time for PDMS/DVB and CAR/PDMS fibers in both liquid and headspace sampling modes are shown in Figure 2. For PDMS/DVB, the adsorptive capacity in both modes reached saturation for diacetyl within 1 min, and saturation with acetoin was reached within 5 min. With the CAR/PDMS fiber, in both modes, saturation with diacetyl was attained within 30 min, but saturation with acetoin was not attained even after 90 min. Therefore, unless otherwise indicated, fibers were exposed for 10 min.

Ionic Strength. As reported previously (Yang and Peppard, 1994) for a wide range of volatiles, the pres-

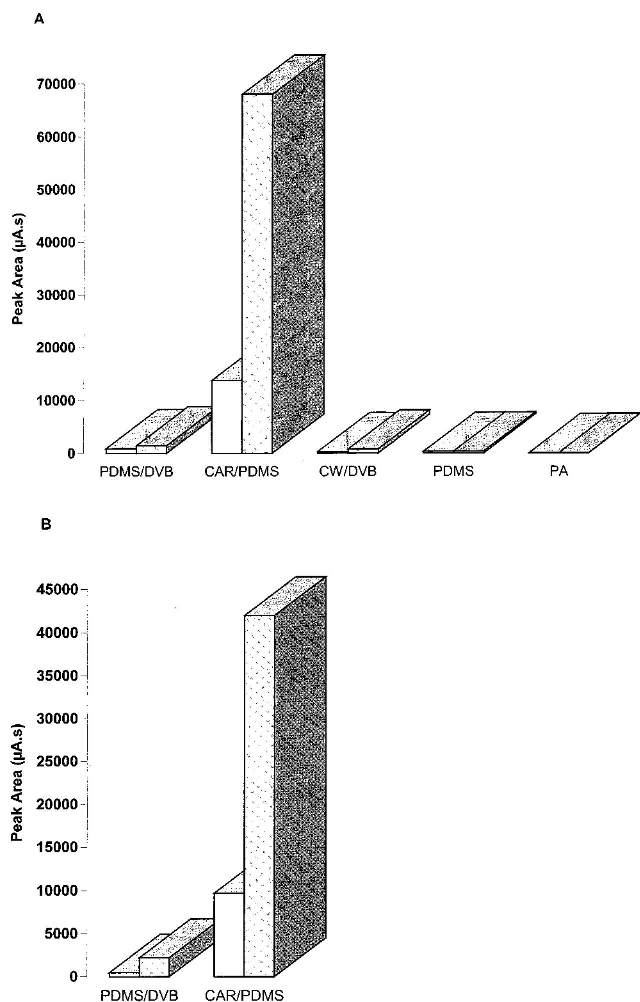

Figure 1. Effect of fiber coating phase on the absorptive capacity of the SPME fibers for acetoin (left) and diacetyl (right). (A) in liquid sampling mode; (B) in headspace sampling mode.

ence of an electrolyte in solution changes the partition behavior of the dissolved compounds. Acetoin and diacetyl are found to behave as type b compounds (as defined by Yang and Peppard, 1994), the adsorption of diacetyl increasing 2.3-fold with salt concentration before leveling off at saturation (data not shown). In liquid sampling, both compounds show a higher level of adsorption when the solution is saturated with NaCl (Figure 2A,B). Analysis in headspace mode was only conducted with the sample saturated with NaCl.

For both fibers, the maximum adsorption is more efficient in LS when the sample is saturated with NaCl. However, HS is more efficient than LS when the sample solution in LS does not contain added salt (Figure 2). In HS, the maximum adsorption with diacetyl is attained more rapidly than in LS, while acetoin shows a more rapid adsorption in LS than in HS. This effect reflects the relative volatility of these two products. Although in LS mode the salt enhances adsorption, an accumulation of salt inside the injection sleeve leads to signal degradation, making HS the better mode for the present application (see below).

Other Parameters. In the GC injector, there is a temperature gradient. Like Arthur et al. (1992a), it was found that there is an optimal position of the fiber inside the injector to obtain the best analyte desorption. In the configuration used, the maximal desorption was obtained with 3.0 cm of injection depth (e.g., 4.5 cm needle + fiber). In contrast, the desorption time, that is, the period during which the fiber is left inside the injector, had no effect on the amount of analyte desorbed, as reported previously for other organic compounds (Gorecki and Pawliszyn, 1995). Using the described experimental conditions, complete desorption occurred in a few seconds.

Table 1. Measurement of the Effect of SPME-GC-C IRMS in HS and in LS on the δ^{13}C Values As Compared with Encapsulated Standards[a]

		diacetyl (−25.4‰)			acetoin (−23.1‰)		
		δ^{13}C (‰) mean	SD	*N*	δ^{13}C (‰) mean	SD	*N*
PDMS/DVB	HS	−26.32	0.18	9	−23.82	0.18	9
	LS	−26.22	0.27	8	−23.56	0.30	8
CAR/PDMS	HS	−26.69	0.12	7	−23.61	0.32	8
	LS	−26.46	0.32	7	−23.78	0.26	7

[a] δ^{13}C values for diacetyl and acetoin obtained by encapsulation are given at the column headings. Values measured by SPME-GC-IRMS represent the mean of *N* determinations made in various conditions of concentration and exposure period.

It has been shown (Yang and Peppard, 1994; Gorecki and Pawliszyn, 1997) that the sample volume can have an influence on the quantification and on the precision of the results. Consequently, the amount of analyte adsorbed by the fiber cannot be neglected, especially if a small sample volume is used. To ensure uniformity in the measurements, a large volume of sample was used (see above), to avoid the risk of analyte exhaustion due to extraction (especially in very weakly concentrated samples) and to permit several IRMS analyses on the same sample.

Magnetic stirring was used during analysis to obtain an homogeneous sample. Inhomogeneity could lead to a diffusion-regulated adsorption which in turn will lead to an isotopic fractionation.

IRMS Analysis. Dias and Freeman (1997), working in LS mode, have shown that isotopic fractionation, measured as δ^{13}C values, for a range of hydrocarbons and organic acids did not vary systematically with extraction time. They found, however, that the δ^{13}C values measured by SPME-GC-C-IRMS were not the same as obtained by encapsulation and that the difference, $\Delta\delta^{13}$C, varied with the nature of the product and, in some cases, with concentration. Therefore, these parameters need to be verified for each class of product studied. In addition, the use of SPME in HS mode linked to an IRMS for the determination of δ^{13}C values has not been reported previously.

Acetoin and diacetyl were extracted from standard solutions with either the PDMS/DVB or the CAR/PDMS fiber. As can be seen from Table 1 and Figures 3 and 4, the introduction of the products extracted with the fibers into the IRMS by SPME-GC-C can lead to a small but significant $\Delta\delta^{13}$C (‰), the extent of which apparently depends on the product. For PDMS/DVB as well as CAR/PDMS, isotopic depletions were observed in the extracted materials. For both fibers, the isotopic fractionation is nearly identical but differs slightly depending on the compound (up to 0.7‰ for acetoin and 1.3‰ for diacetyl). Isotopic data obtained for acetoin and diacetyl with the PDMS/DVB or the CAR/PDMS fiber falls within limits of ±0.4 (‰). This limit has been defined as the maximum acceptable standard deviation for the mean value obtained for 10 sequential injections of standard solutions into the GC-C-IRMS.

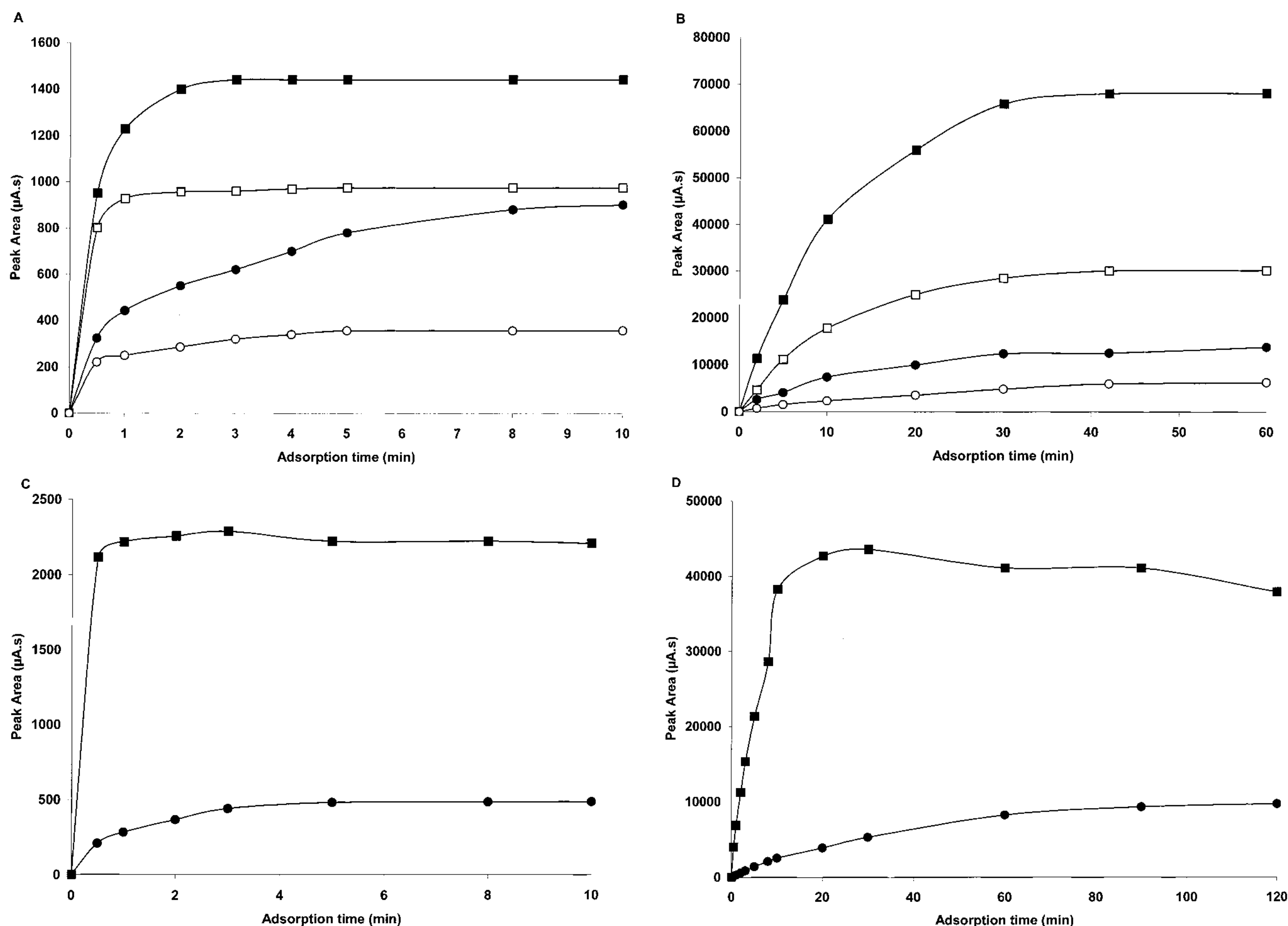

Figure 2. Kinetics of adsorption of acetoin and diacetyl by the fibers. (A) PDMS/DVB in liquid sampling mode; (B) CAR/PDMS in liquid sampling mode; (C) PDMS/DVB in headspace sampling mode; (D) CAR/PDMS in headspace sampling mode; with (solid symbols) and without (open symbols) saturating NaCl; acetoin (circles), diacetyl (squares).

Effect of the SPME Sampling Mode on the $\delta^{13}C$ Values. Similarly, a preliminary assessment of the sources of isotopic fractionation in LS and HS mode was carried out for acetoin and diacetyl extracted from standard solutions (Table 1, Figures 3 and 4). No significant differences were observed between sampling modes, regardless of the fiber type or the nature of the extracted compound.

Effect of the Concentration of Analytes on $\delta^{13}C$ Values. During the fermentation, the concentrations of acetoin and diacetyl can vary between zero and approximately 4 mM. Therefore, it was necessary to evaluate the stability of the δ^{13}C values obtained over a range of conditions equivalent to those found during a bacterial fermentation. These analyses were done with both the PDMS/DVB and the CAR/PDMS fibers. Figure 3 shows the effect of varying the concentration of acetoin and diacetyl on the δ^{13}C values obtained in LS and HS mode. When the fiber was exposed for 10 min, the IRMS spectrometer is saturated above 1.2 mM with the PDMS/DVB fiber and above 0.12 mM with the CAR/PDMS fiber. Within these limits, however, it is clear that there is no progressive effect of concentration on the δ^{13}C values obtained in either sampling mode.

Thus, the data show that the determined δ^{13}C values for acetoin and diacetyl are within experimental error over the entire range of concentration that can be studied. However, at higher concentrations with a 10-min exposure, the fiber adsorbs too much diacetyl (above 1.2 mM), leading to saturation of the IRMS detector. This problem could be avoided either by dilution of the sample or by using a shorter exposure period.

Effect of the Period of Fiber Exposure on $\delta^{13}C$ Values. As seen previously, the period of exposure has an effect on the amount of adsorbed analyte. This in turn could influence the measured δ^{13}C values, making it difficult to compare data obtained from, for example, high and low concentrations or with different fibers. The effect of exposure times between 1 and 10 min with both PDMS/DVB and CAR/PDMS on the δ^{13}C values obtained for diacetyl and acetoin are given in Figure 4. Within this time domain, it is clear that the δ^{13}C values obtained show no consistent variation in either sampling mode. Repeatability was found to be acceptable, falling within limits of $\pm$0.4 (‰).

Fermentation Analysis. To assess the application of SPME-GC-C-IRMS to the analysis of acetoin and diacetyl in fermentations, a series of fermentations were carried out. Initially, a fermentation with only glucose (5 g/L) as carbon source was performed. A GC analysis by SPME of this medium, saturated with NaCl, showed no interfering compounds present in the diacetyl and acetoin domain (data not shown). Subsequently, acetoin ($\delta^{13}C = -23.1$ ‰) and diacetyl ($\delta^{13}C = -25.4$ ‰) were added. The acetoin and diacetyl were recovered from the spiked fermentation medium by SPME in both sampling modes. The values obtained are identical to those expected within the $\pm$0.4 ‰ precision, showing that there is no influence of the medium on the isotopic values obtained in SPME-GC-C-IRMS. Thus, it can be

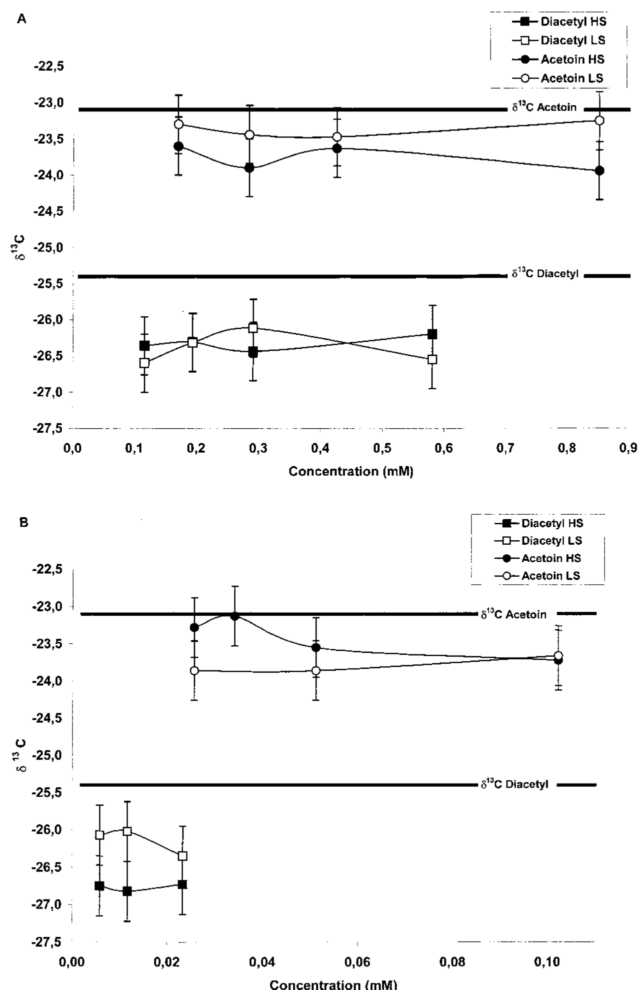

Figure 3. Effect of concentration of acetoin and diacetyl on the isotopic values in ^{13}C determined by SPME-GC-C-IRMS. Values for ^{13}C are expressed on the relative δ^{13}C (‰) scale, with Pee Dee Belamnite as international reference. Values of δ^{13}C obtained for encapsulated acetoin and diacetyl are shown as the solid bars. (A) PDMS/DVB fiber; (B) CAR/PDMS fiber; open symbols = LS mode; closed symbols = HS mode; acetoin (circles), diacetyl (squares). Each value represents the mean of at least three independent measurements within a standard deviation of less than 0.4 (‰). The acceptable range of measurements (±0.4 ‰, see text) is shown by the vertical bars.

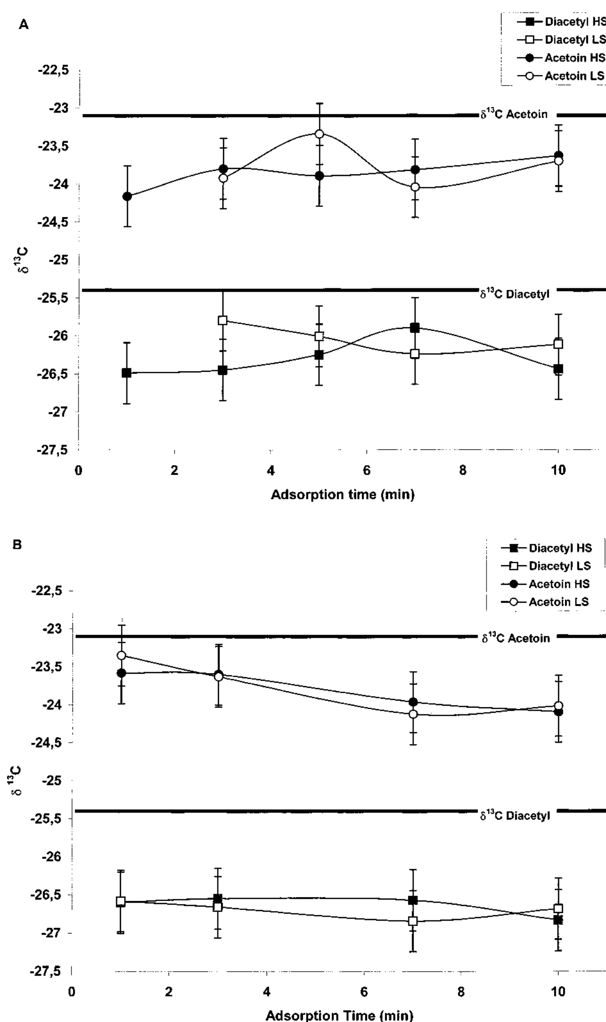

Figure 4. Effect of the period of exposure of the fiber to acetoin and diacetyl on the isotopic values in ^{13}C determined by SPME-GC-C-IRMS. Values for ^{13}C are expressed on the relative δ^{13}C (‰) scale, with Pee Dee Belamnite as international reference. Values of δ^{13}C obtained for encapsulated acetoin and diacetyl are shown as the solid bars. (A) PDMS/DVB fiber (diacetyl, 0.30 mM; acetoin, 0.43 mM); (B) CAR/PDMS fiber (diacetyl, 0.01 mM; acetoin, 0.20 mM); open symbols = LS mode; closed symbols = HS mode; acetoin (circles), diacetyl (squares). Each value represents the mean of at least three independent measurements within a standard deviation of less than 0.4 (‰). The acceptable range of measurements (±0.4 ‰, see text) is shown by the vertical bars.

concluded that the medium contains no other products that interfere with the chromatographic separation of these compounds after they have been adsorbed on the SPME fibers.

A second fermentation, with glucose and citric acid, was analyzed for the δ^{13}C values of the endogenous acetoin and diacetyl after 16 h of fermentation, previously found to be the maximal production of these compounds under these culture conditions (Goupry et al., 2000). The δ^{13}C values obtained (Table 2) are intermediate to those from the δ^{13}C values for the initial glucose and citric acid, showing a clear utilization of both substrates for the biosynthesis of the aroma compounds. Equivalent data were obtained using either LS or HS mode.

Table 2. Measurement by SPME-GC-C-IRMS in HS of the δ^{13}C Values of Acetoin and Diacetyl Obtained by Fermentation of Glucose and Citric Acid[a]

	diacetyl			acetoin		
	δ^{13}C (‰) mean	SD	*N*	δ^{13}C (‰) mean	SD	*N*
PDMS/DVB	−18.7	0.36	3	−18.4	0.16	3
CAR/PDMS	−18.9	0.16	3	−18.5	0.05	3

[a] Initial values of δ^{13}C for glucose (5 g/L) and citric acid (2.5 g/L) were −10.0 and −23.6, respectively. Measured values are not corrected for the effect of the SPME fiber.

CONCLUSION

It is demonstrated for the first time that the SPME-GC-C-IRMS method can be applied to the extraction and the determination of the δ^{13}C values of acetoin and diacetyl from fermentation media. Two type of fibers are shown to be suitable. Both of these cause a small but consistent $\Delta\delta^{13}$C with reference to the δ^{13}C values determined by encapsulation, as previously reported (Dias and Freeman, 1997). Therefore, as the concentration and the period of exposure have no influence on the δ^{13}C values measured in either LS or HS mode, the SPME-GC-C-IRMS method can be applied

to the extraction and the isotopic analysis of these metabolites within acceptable limits of ±0.4 (‰). The analytical conditions can be varied to ensure that the sample is applied to the IRMS spectrometer within the permitted range of sensitivity by using long exposure times with a CAR/PDMS fiber for dilute samples (≤0.1 mM) or by using short exposure times with a PDMS/DVB fiber for concentrated samples (≤2 mM). Because of the variable conditions found in fermentation media, it is desirable to be able to work with different extraction regimes, each suited to the particular needs of analysis. The data presented here show that it is not necessary to wait for equilibrium to be reached to obtain an acceptable value. This technique has been successfully used to study the co-metabolism of citrate and glucose into acetoin and diacetyl during fermentation by *L. lactis* ssp. *lactis* biovar. *diacetylactis* (Goupry et al., 2000).

ABBREVIATIONS USED

CAR/PDMS, carboxene/poly(dimethylsiloxane); CW/DVB, carbowax/divinylbenzene; GC-C-IRMS, gas chromatography−combustion interface−isotope ratio mass spectrometry; GC, gas chromatography; HS, headspace sampling; IRMS, isotope ratio mass spectrometry; LAB, lactic acid bacteria; LS, liquid sampling; PA, polyacrylate; PDMS/DVB, poly(dimethylsiloxane)/divinylbenzene; PDMS, poly(dimethylsiloxane); SPME, solid-phase microextraction.

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